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Short Communication

Fractionation of Stable Isotopes of Nitrogen in *Trapa japonica* During Uptake of NH_4^+-N

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Trapa japonica **grown hydroponically under various conditions with respect to different concentrations of NH_4^+-N to observe the fractionation of stable isotope of nitrogen occurred during uptake and assimilation. There was a significant variation in fractionation of nitrogen isotope during uptake from different levels of nitrogen by the plant. When plant absorbed a large amount of N from the solution, the lower enrichment of $\delta^{15}N$ value was observed in plant parts and the enrichment of $\delta^{15}N$ value was decreased with the increase of nitrogen concentration in the medium. A linear relationship occurred during up take of nitrogen by *T. japonica* between fraction of nitrogen remaining in medium (*f*) and fractionation factor (ε).**

Keywords: *Trapa japonica;* Fractionation; Nitrogen isotopes,

Trapa japonica is an annual floating leaved hydrophyte typical of natural wetlands. The natural variation in stable isotopes has been shown to be a powerful tool in several studies of plant ecosystem N dynamics (Handley and Raven, 1992; Page, 1995; Hietz *et al.*, 2002). The natural abundance of ^{15}N ($\delta^{15}N$) of plant biomass has been shown to vary as a function of isotopic composition of primary N source (soil, fertilizer, N_2) (Shearer and Kohl, 1986; Johannisson and Högberg, 1994) and the isotope ratio of the source is preserved during N absorption, assimilation and translocation. However, physico-chemical processes in soil like N-uptake, denitrification by bacteria, leaching and volatilization of organic nitrogen compounds and physiological processes within plant like assimilation through distinct pathways can discriminate against ^{15}N (Robinson, 2001; Ariz *et al.*, 2011). Therefore, $\delta^{15}N$ of plants reflects the net effect of a range of processes (Evans, 2001; Robinson, 2001).

Remarkably little is known about whether fractionation occurs during the uptake and assimilation of N from different concentration by *T. japonica* and the relationship between uptake rate and fractionation. In the present study, we hypothesized that there would be no discrimination against ^{15}N in *T. japonica* fed with different levels of NH_4^+-N. Therefore, fractionation of stable N isotopes, if any, will provide insight into the response of this species to different levels of N. The objective of this study was therefore; to investigate the fractionation of stable N isotopes occurred during uptake as a function of nitrogen levels.

Materials and methods

Experimental setup

In this study, the plants were grown in 2 L plastic beaker with NH_4^+-N of five concentrations (0, 10, 20, 40, 80 ppm). The sources of N was $(NH_4)_2SO_4$. Approximately 400 g of commercial river sand (90% < 1mm) (DIY, Doit, Japan) were used as substrate in each beaker. Before placing sand in the beaker, the sand was thoroughly washed with tap water until the supernatant appeared clear and free of loose particles. Finally, the sand was washed with distilled water. Washing the substrate ensured that it did not provide any nutrients that could interfere with the

effect of supplied N on the growth of *T. japonica* in the beakers. Since N was used as a treatment, a modified Hoagland solution (10%) (Hoagland and Arnon, 1950) was used as a cultural medium, where $Ca(NO_3).2H_2O$ and KNO_3 were replaced by $CaCl_2.2H_2O$ and KCl, respectively. 1.8 L of culture medium was used in each beaker and the volume was adjusted biweekly with distilled water to compensate for evapotranspiration. The pH of the solutions were maintained at 7.0 ± 0.5 by adding HCl or NaOH biweekly. Seeds of *T. japonica* were collected from a naturally growing population at Oaso Park, Kumagaya city, Japan in December 2010. Seeds were chilled at temperature 4° C for 30 days. The healthy seeds of *T. japonica* were placed in 2 L beakers at the end of March 2010.

The experiment was conducted for a period of 3 weeks repeated twice in growth chamber with a constant temperature of 20°C. Illumination was supplied using 4×20W fluorescent lamps (Kyushu Denki Hanabai Corporation, Japan) with a photoperiod of 12 hrs light and 12 hrs dark.

Plant analysis

At the end of 3 weeks, plants from beakers were harvested, cleaned, separated different parts and sorted into leaves, stems, water roots and soil roots. The plant parts were air dried for 24 hours followed by an oven at 60°C to constant weights. Following drying, samples were re-weighed (to obtain a final dry mass) then were homogenized by grinding into fine powder using a mortar and pestle. Powdered samples were stored in air-tight vials for subsequent analyses.

Water analyses

Water sample was collected from each beaker after immediately placing the seeds of *T. japonica* in the beakers and at harvesting time. After every collection, it was filtered through Whatman42 filter paper and stored in refrigerator (4° C) until analyses. NH_4^+-N and NO_3^--N of water were measured using an auto analyzer (TRAACS 800, Technicon, New York, USA). Total nitrogen (TN) was measured by UV spectrophotometric screening method (APHA, 1998).

Stable isotope ratios

Nitrogen stable isotope ratio, $\delta^{15}N$ (‰) was determined separately for tissues of different plant organs. The combustion product, N_2 was introduced into an isotope-ratio mass spectrometer (model: Isoprime, Micromass UK) in a continuous flow using a He carrier. Ratios of ^{15}N:^{14}N were expressed relative to the PeeDee Belemnite (PDB) standard for N_2 in air for N. The ratio, ^{15}N:^{14}N, was calculated as

$$\delta^{15}N = \left[\left(\frac{R_{sample}}{R_{reference}}\right) - 1\right] \quad (1)$$

where, $R = {}^{15}N/{}^{14}N$

For the determination of $\delta^{15}N$ of N in water samples, the simple and rapid method developed by Sakata (2001) was used. In this method, NH_4^+ and NO_3^- in water samples were separated as NH_4^+ into diluted H_2SO_4 by sequential distillation techniques. Then NH_4^+ in the distillate was precipitated directly as insoluble salt of $(C_6H_5)_4BNH_4$, which was subsequently combusted in the above mentioned isotope-ratio mass spectrometer. All samples were tested more than twice with standard deviations (S.D.) being < 0.05 for $\delta^{15}N$ (‰).

Statistics analyses

All data are presented in the paper as mean with Standard Deviation (SD). Differences among treatments were analyzed by one-way ANOVA to check the significant differences ($P < 0.05$) with a post hoc Turkey test. For this purpose SPSS for windows (release 13, SPSS INC., Chicago, IL) statistical software package was used.

Theory

The abundance of heavy isotope is referred to that of the lighter one. Due to the small abundance of ^{15}N in atmospheric nitrogen and the small variation of ^{15}N abundance in the nitrogen cycle, $R=^{15}N/^{14}N \sim {}^{15}N/(^{14}N+{}^{15}N)$, approximately holds, where the non-dimensional parameter, $R = {}^{15}N/{}^{14}N$, is used. The relative unit is, then, defined as

$$\delta^{15}N\ (‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000 \quad (2)$$

Mariotti et al. (1981) provided the relation between the ratio of the nitrogen abundance with respect to the original value as

$$\frac{\varepsilon}{1000}\ln f = \ln\frac{R_w}{R_{wo}} = \ln\frac{10^{-3}\delta^{15}N_w+1}{10^{-3}\delta^{15}N_{W_0}+1} \qquad (3)$$

Where, $f = N_w/N_{W_0}$, approximately equal to the ratio of the nitrogen abundance in the surrounding water with respect to the original value and subscript w_0 and w indicates the value of the original water (initial water) and at a specific time (harvest time), respectively. The fractionation factor, ε , is given by

$$\varepsilon = 1000\ln\frac{10^{-3}\delta^{15}N_w+1}{10^{-3}\delta^{15}N_{wo}+1}/\ln f \qquad (4)$$

The balance of isotropic abundance provides the isotopic ratio of the nitrogen taken up by the plant as

$$^{15}N_{up} = 1000\left(\left(1+\frac{\delta^{15}N_{wo}}{1000}\right)\frac{1-f^{1+\frac{e}{1000}}}{1-f}-1\right) \qquad (5)$$

Nitrogen content of the plant (N_{pl}) is attributable to the endosperm in the seed, while N_{es} is nitrogen taken up from endosperm and N_{up} is the nitrogen taken up from the surrounding water. Therefore,

$$N_{pl} = N_{es} + N_{up} \quad \text{(or)}$$

$$^{15}N_{pl}\,N_{pl} = {}^{15}N_{es}N_{es} + {}^{15}N_{up}N_{up} \qquad (6)$$

Correspondingly, ^{15}N of the plant, $^{15}N_{pl}$ is given by

$$^{15}N_{pl} = \frac{{}^{15}N_{es}N_{es}+{}^{15}N_{up}N_{up}}{N_{pl}} \qquad \text{or,}$$

$$^{15}N_{up} = \frac{1}{N_{up}}({}^{15}N_{pl}N_{pl} - {}^{15}N_{es}N_{es}) \qquad (7)$$

Results and Discussion

Figure 1 shows the $\delta^{15}N$ of plant tissues after 3 weeks treatment. The $\delta^{15}N$ value of added ammonium was -10.84 ‰, while the value of $\delta^{15}N$ of the water gradually rose up as ^{14}N was discriminatively taken up by plants. At the same time, $\delta^{15}N$ values of the plant declined. The $\delta^{15}N$ of seed endosperms was 7.1 ± 0.06 (‰). The $\delta^{15}N$ value was lower in plant parts with increasing nitrogen concentration in the media. Among tissues, the $\delta^{15}N$ was the highest in leaves, followed by stems and lowest was in roots, regardless of concentration of nitrogen in water. The fractionation of stable isotope of nitrogen is related to concentration of nitrogen in the medium (fig. 2). There was linear relationship between fractionation factor and fraction of nitrogen remaining in the medium (fig. 3).

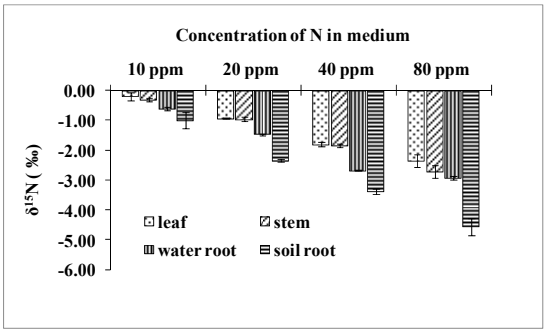

Fig. 1 Effect of nitrogen on $\delta^{15}N$ value in different parts of plant

The highest enrichment for ^{15}N was observed in the leaves followed by stems and the lowest enrichment was in soil roots followed by water roots when plants were fed with different concentration of nitrogen. NH_3 is lost by stomata at high concentration of nitrogen in plants (Mattsson and Schjoerring, 1996; Mattsson *et al.*, 1998) which may favor the enrichment of ^{15}N in leaves and stems more than in roots (Schjoerring *et al.*, 2000; Massad *et al.*, 2010; Ariz, 2011).

Due to preferential activity, *T. japonica* showed lower enrichment of ^{15}N in higher concentration of nitrogen where the lighter isotope of nitrogen is available. May be for this reason more fractionation is occurred in higher concentration of nitrogen than lower concentration as there is scarcity of ^{14}N (the lighter isotope) in lower concentration of nitrogen of the medium.

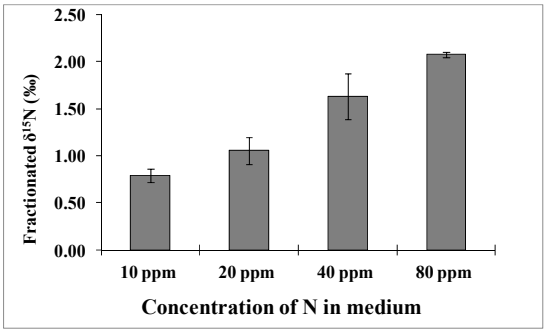

Fig. 2 Fractionation occurred during uptake of nitrogen from the medium

Nitrogen isotopic fractionation against ^{15}N was caused by volatilization of NH_3 in the

aerial parts of plants (O'Deen, 1989). Mariotti et al. (1982) and Bergersen et al.(1988) also observed lower values of $\delta^{15}N$ in the roots than in shoots. The lower negative values of $\delta^{15}N$ in plants were observed may be for losing N from the plant in the form of root efflux and exudates (Evans, 2001; Yoneyama *et al.*, 2001; Kolb and Evans, 2003).

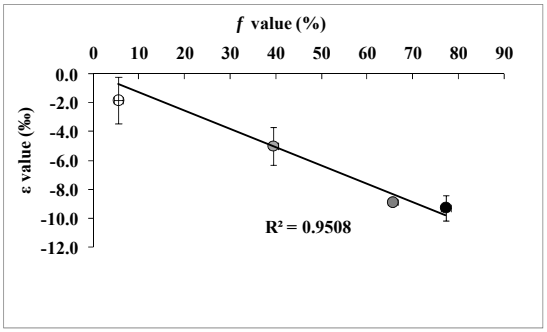

Fig. 3 Relationship between fraction of nitrogen remaining in medium (*f*) and fractionation factor (ε) derived from cultural media of *T. japonica*. Blank to dark in round circle indicates low to high concentration of nitrogen in the medium.

We found that the negative values were increased with the increase of N concentration in the medium. The $\delta^{15}N$ values in plant parts were closer to the source $\delta^{15}N$ in low N availability conditions (at low N concentrations). Likewise, when the N concentration increased, the amount of substrate became unlimited and the isotope effect was observed.

There are several possibilities for the nitrogen deficit; however, the most probable reason is the loss due to de-nitrification. Associated with the nitrogen reduction in the tank, total amount of $\delta^{15}N$ in each tank substantially increased. The $\delta^{15}N$ values changed substantially by the nitrification and de-nitrification processes, thus, the fractionation rate is unable to obtain from the difference in the $\delta^{15}N$ values of water and plants.

According to the equation 7 we calculated the $\delta^{15}N$ in plant on the basis of uptake nitrogen from the medium only. Thus we have got the fractionation occurred during uptake nitrogen by plant from the medium by subtracting the plant $\delta^{15}N$ value obtained only by uptaking N from the medium from the initial $\delta^{15}N$ value of medium (Evans, 2001).

There was a linear relationships between $\ln(f)$ and $1000\ln(R_w/R_{w0})$ which provides the fractionation factors (ε). Figure 3 shows the relationship between ε values and *f* values (the percentage of total added N that remains in the medium). The *f* values can be used as indirect measure of nitrogen uptake rate (Yoneyama et al., 2001). There is a linear relationship between ε and *f* values. It suggests that fractionation occurred in plant is directly related with uptake N from medium.

It can be concluded that there was a significant fractionation was occurred during uptake of N by *Trapa japonica* which depends on concentration of nitrogen in the medium as well as uptake rate of nitrogen by the plant.

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